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Inhibition of aflatoxin biosynthesis by phenolic compounds

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S.-S.T. HUA, O.-K. GROSJEAN AND J.L. BAKER. 1999. The phenolic compounds acetosyringone, syringaldehyde and sinapinic acid inhibited the biosynthesis of aflatoxin B_1 (AFB_1) by *A. flavus*. Acetosyringone was the most active among the three compounds, inhibiting aflatoxin level by 82% at 2 m mol l^{-1}. The synthesis and accumulation of norsolorinic acid, an aflatoxin biosynthetic intermediate, was also inhibited. These results suggest that at least one step early in the AFB_1 biosynthetic pathway is inhibited by the phenolics.

INTRODUCTION

The filamentous fungus *Aspergillus flavus* produces aflatoxin B_1 (AF B_1), a substance highly toxic to mammals and one of the most potent carcinogens known (Ellis *et al*. 1991; Payne 1992; Scudamore 1994). AFB_1 is synthesized through the polyketide pathway, involving more than 25 genes (Bennett *et al*. 1994; Bhatnagar *et al*. 1996). The first stable intermediate is norsolorinic acid (NOR). At least 16 consecutive enzyme-catalysed reactions are required to complete the synthesis of AFB_1 from NOR. The *nor* mutant of *A. flavus* (Bennett 1979; Papa 1984) has a defective norsolorinic acid reductase and thus blocks in the aflatoxin biosynthetic pathway, resulting in the accumulation of NOR, a bright red-orange pigment.

Phenolics are secondary plant metabolites synthesized via the phenylpropanoid biosynthetic pathway. These compounds are building blocks for cell wall structures and serving as defense against pathogens (Hahlbrock and Scheel 1989). In this study, the effects of the three phenolics acetosyringone, sinapinic acid and syringaldehyde on aflatoxin biosynthesis in *A. flavus* are evaluated. The *nor* mutant of *A. flavus* Papa 827 was used to examine the effect of these compounds on NOR accumulation, an early step in aflatoxin biosynthesis.

MATERIALS AND METHODS

Fungal cultures

The toxigenic strain *A. flavus* 42–12 (NRRL-25347) was isolated from pistachio nuts. This strain produces AFB_1 and traces of AFB_2. *A. flavus* 827 Papa, a white-spored *nor* mutant (Papa 1984) was obtained from N. Keller, Texas A&M University. The fungal strains were maintained on potato-dextrose agar (PDA). Fungal spores were suspended in Tween-80 peptone water (0·5 ml/l Tween 80, 1 g/l peptone) and their number was determined microscopically using a haemocytometer.

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Effect of phenolic on aflatoxin production

Acetosyringone (3,5-Dimethoxy-4-Hydroxyacetophenone) was purchased from Aldrich Chemical Company, Milwaukee, WI and syringaldehyde and sinapinic acid from Sigma Chemical Co., St Louis, MO. Potato dextrose agar (PDA) was purchased from DIFCO Laboratories, Detroit, MI. Phenolic compounds were incorporated into PDA to final concentrations of 1, 2, 3 and 4 m mol l^{-1}. PDA (5 ml) was dispensed into each Petri dish (60 mm × 10 mm). Each of triplicate Petri dishes was inoculated with 5 μl of a spore suspension (1×10^5 spores/ml), then incubated at 28 °C in the dark for 10 d. All the experiments were repeated twice. Aflatoxin was extracted and analysed by high pressure liquid chromatography (HPLC) on a Hewlett Packard model 1050 Chem Station, Hewlett-Packard, Palo Alto, CA, USA (Rodriguez and Mahoney 1994; Hua *et al*. 1999).

Determination of norsolorinic acid

The *nor* mutant was cultured in PDA containing phenolics at concentrations from 1 to 4 m mol l^{-1}. The fungal culture, including agar from the entire Petri dish, were transferred to a centrifuge tube. The bright red-orange pigment in the mycelia was extracted by 15 ml of alkaline methanol. NOR

Table 1 Effect of phenolic compounds on norsolorinic acid accumulation*

Phenolics	Concentration (m mol l^{-1}) 0	1	2	3	4
Acetosyringone	1·66 ± 0·21	0·92 ± 0·12	0·58 ± 0·07	0·32 ± 0·04	0·17 ± 0·02
Syringaldehyde	1·66 ± 0·21	1·33 ± 0·16	1·06 ± 0·11	0·71 ± 0·08	0·54 ± 0·07
Sinapinic acid	1·66 ± 0·21	1·42 ± 0·18	1·28 ± 0·15	1·22 ± 0·16	1·16 ± 0·14

*The amount of norsolorinic acid accumulated per fungal culture in each Petri dish is expressed in mg.
Values are means of six independent samples ± standard deviation.

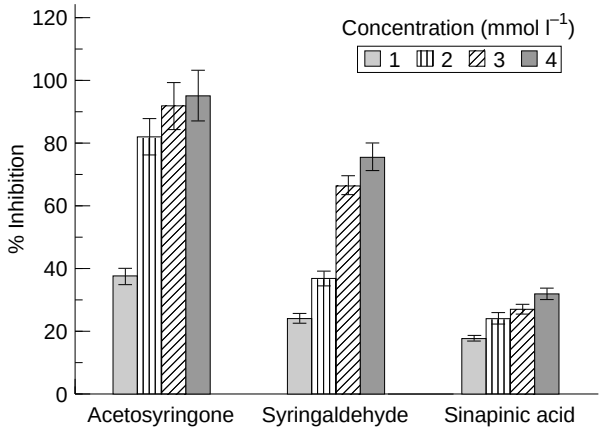

Fig. 1 Inhibition of aflatoxin B_1 biosynthesis by phenolics. The bar represents the mean percentage of inhibition by each phenolic at concentrations ranging from 1 to 4 m mol/l. Values are means ± standard deviations ($n = 6$) of two sets of triplicate samples from two independent experiments

concentration in the supernatant fluid was determined spectrophotometrically at a wavelength of 560 nm in a Beckman DU 640 spectrophotometer Beckman Coulter Inc., Fullerton, CA, USA (Hua *et al.* 1999). Purified NOR was dissolved in alkaline methanol and used as standards.

RESULTS AND DISCUSSION

All three phenolics inhibited AFB_1 biosynthesis by *A. flavus*. The order of inhibitory activity was acetosyringone > syringaldehyde > sinapinic acid (Fig. 1). A concentration of 1 m mol/l acetosyringone in the PDA media resulted in about a 38% reduction of AFB_1 levels. The concentration of acetosyringone necessary to achieve 82% inhibition of aflatoxin biosynthesis is 2 m mol l^{-1}. Increasing acetosyringone concentration to 4 m mol l^{-1}, reduced aflatoxin biosynthesis by 96%. In the case of syringaldehyde, about 37% inhibition of aflatoxin biosynthesis was observed at a concentration of 2 m mol l^{-1} in the media. When the concentration of syringaldehyde was raised to 4 m mol l^{-1} in PDA, an inhibition of 75% was achieved. Sinapinic acid showed the least inhibitory activity on aflatoxin biosynthesis, only 31% of inhibition was observed at a concentration of **4** m mol l^{-1}.

The inhibitory effect of these phenolics on NOR accumulation in the *nor* mutant was quickly and easily visualized by the intensity of the red-orange colour in the agar cultures. As the concentrations of acetosyringone in the growth media were increased from 1 to 4 m mol l^{-1}, the intensity of the red orange colour decreased considerably. A less dramatic inhibition of pigmentation was observed with sinapinic acid and syringaldehyde. The concentrations of NOR accumulated under the influence of these three phenolics are summarized in Table 1.

The results demonstrated that the three phenolic compounds not only inhibited aflatoxin biosynthesis in an aflatoxigenic strain of *A. flavus*, but also reduced NOR accumulation in the *nor* mutant. These compounds inhibited one or more early rather than late steps in the pathway, so accumulation of toxic intermediates formed in the later steps will not occur. Utilization of these phenolic compounds to reduce aflatoxin contamination in food and feeding stuffs warrants further research.

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